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2016 Protocols

Illumina MiSeq Metagenomic Sequencing

Adapted from:
Nextera XT DNA Sample Preparation Guide. (2012). Illumina.

Purpose:
To sequence whole genome microbial DNA isolated from animal fecal matter and perform functional analysis of bacterial communities.

Reference:
This protocol was adapted from:
Nextera® XT DNA Sample Preparation Guide. (2012). *Illumina.*

Required Materials:

- Nextera XT DNA Sample Preparation Kit
- Nextera XT DNA Sample Preparation Index Kit
- Multichannel pipettes (10 – 1000 μL)
- Single channel pipettes (10 – 1000 μL)
- Pipette tips (10 – 1000 μL)
- 96-well storage plates, round well, 0.8 ml ("MIDI" plate)
- Agencourt AMPure XP 60 ml kit
- Distilled water
- Ethanol 200 proof (absolute) for molecular biology (500 ml)
- Microseal 'A' film
- Microseal 'B' adhesive seals
- PCR grade water (for gel-free method)
- RNase/DNase-free multichannel reagent reservoirs, disposable
- Tween 20
- Ultra pure water
- Microseal 96-well PCR plates ("TCY" plate)
- 96-well thermal cycler (with heated lid)
- Heat Block for 1.5 ml centrifuge tubes
- High Speed Micro Plate Shaker
- Magnetic stand-96
- Microplate centrifuge
- Vortexer

Procedure:
1. Label a new 96-well TCY plate **NTA** (Nextera XT Tagment Amplicon Plate).
2. Add 10 μL of thawed Tagment DNA (TD) Buffer to each well to be used in this assay. Change tips between samples.
3. Add 5 μL of input DNA at 0.2 ng/μL (1 ng total) to each sample well of the **NTA**

plate.

4. Add 5 μL of Amplicon Tagment Mix (ATM) to the wells containing input DNA and TD Buffer. Change tips between samples.
5. Using a multichannel pipette, gently pipette up and down 5 times to mix. Change tips between samples.
6. Cover the **NTA** plate with Microseal 'B'.
7. Centrifuge at 280 xg at 20°C for 1 minutes.
8. Plate the **NTA** plate in a thermocycler and run the following program:
    - 55°C for 5 minutes
    - Hold at 10°C
9. Once the sample reaches 10°C, remove from the thermocycler.
10. Remove the Microseal 'B' seal and add 5 μL of Neutralize Tagment (NT) Buffer to each well of the **NTA** plate. Change tips between samples.
11. Using a multichannel pipette, gently pipette up and down 5 times to mix. Change tips between samples
12. Cover the **NTA** plate with Microseal 'B'.
13. Centrifuge at 280 xg at 20°C for 1 minute.
14. Place the **NTA** plate at room temperature for 5 minutes
15. Arrange the index primers in the TruSeq Index Plate Fixture using the following arrangement:
    - Arrange index 1 (i7) primer tubes (orange caps) in order horizontally, so the N701 is in column 1 and N712 is in column 12.
    - Arrange index 2 (i5) primers (white caps) in order vertically, so that S501 is in row A and S508 is in row H.
    - Record their positions on the lab tracking form.
16. Plate the **NTA** plate in the TruSeq Index Plate Fixture.
17. Add 15 μL of Nextera PCR Master Mix (NPM) to each well of the **NTA** plate containing index primers. Change tips between samples.
18. Using a multichannel pipette, add 5 μL of index 2 primers (white caps) to each column of the **NTA** plate.
19. Using a multichannel pipette, add 5 μL of index 1 primers (orange caps) to each row of the **NTA** plate.
20. Using a multichannel pipette, gently pipette up and down 5 times to mix. Change tips between samples.
21. Cover the plate with Microseal 'A' and seal with a rubber roller.
22. Centrifuge at 280 xg at 20°C for 1 minute.

23. Perform PCR using the following program on a thermal cycler:

	Temperature	Time
Heating	72°C	3 min
Initial Denaturation	95°C	30 s
12 cycles of:	95°C	10 s
	55°C	30 s
	72°C	30 s
Final Extension	72°C	5 min
Hold	10°C	∞

24. Centrifuge the **NTA** plate at 280 xg for 1 min (20°C) to collect condensation.
25. Label a new MIDI plate **CAA** (Clean Amplified Plate).
26. Using a multichannel pipette set to 50 μL, transfer the PCR product from the **NTA** plate to the **CAA** plate. Change tips between samples.
27. Vortex the AMPure XP beads for 30 seconds to ensure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.
28. Using a multichannel pipette, add 30 μL of AMPure XP beads to each well of the **CAA** plate.
29. Gently pipette mix up and down 10 times.
30. Incubate at room temperature without shaking for 5 minutes.
31. Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
32. With the **CAA** plate on the magnetic stand, use a multichannel pipette to carefully remove and discard the supernatant. Change tips between samples.
33. With the **CAA** plate on the magnetic stand, wash the beads twice with freshly prepared 80% ethanol.
34. With the **CAA** plate on the magnetic stand, allow the beads to air-dry for 15 minutes.
35. Remove the **CAA** plate from the magnetic stand. Using a multichannel pipette, add 52.5 μL of Resuspension Buffer (RSB) to each well of the **CAA** plate.
36. Gently pipette mix up and down 10 times, changing tips after each column.
37. Incubate at room temperature for 2 minutes
38. Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
39. Label a new TCY plate **CAN** (Clean Amplified NTA Plate).
40. Using a multichannel pipette, carefully transfer 50 μL of the supernatant from the **CAA** plate to the **CAN** plate. Change tips between samples.
41. Label a new MIDI plate **LNP** (Library Normalization Plate).
42. Using a P20 multichannel pipette and fine tips, carefully transfer 20 μL of the supernatant from the **CAN** plate to the **LNP** plate. Change tips between samples to avoid cross-contamination.
43. For 96-samples, add 4.4 ml of Library Normalization Additives 1 (LNA1) to a fresh

15 ml conical tube.

44. Use a P1000 pipette set to 1000 μL to resuspend Library Normalization Beads 1 (LNB1) thoroughly by pipetting up and down 15-20 times, until the bead pellet at the bottom is completely resuspended.
45. Immediately after LNB1 is thoroughly resuspended, use a P1000 pipette to transfer 800 μL of LNB1 to the 15 ml conical tube containing LNA1. Mix well by inverting the tube 15-20 times. Pour the bead mix into a trough and use it immediately in the next step.
46. Using a multichannel pipette, add 45 μL of the combined LNA1/LNB1 to each well of the **LNP** plate containing libraries.
47. Seal the **LNP** plate with Microseal 'B'.
48. Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 30 minutes.
49. Place the plate on a magnetic stand for 2 minutes and confirm that the supernatant has cleared.
50. With the **LNP** plate on the magnetic stand, using a multichannel pipette set to 80 μl carefully remove and discard the supernatant in an appropriate hazardous waste container.
51. Remove the **LNP** plate from the magnetic stand and wash the beads with Library Normalization Wash 1 (LNW1) as follows:
    - Using a multichannel pipette, add 45 μl of LNW1 to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.
    - Seal the **LNP** plate with Microseal 'B'.
    - Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
    - Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
    - Carefully remove and discard the supernatant in an appropriate hazardous waste container.

52. Remove the **LNP** plate from the magnetic stand and repeat the wash with LNW1 as above.
53. Remove the **LNP** plate from the magnetic stand and add 30 μl of 0.1 N NaOH (less than a week old) to each well to elute the sample.
54. Seal the **LNP** plate with Microseal 'B'.
55. Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
56. During the 5 minute elution, apply the **SGP** (StoraGe Plate) barcode plate sticker to a new 96-well PCR plate.
57. Add 30 μl Library Normalization Storage Buffer (LNS1) to each well to be used in the SGP plate.
58. After the 5 minute elution, ensure all samples in the **LNP** plate are completely resuspended. If the samples are not completely resuspended, gently pipette those samples up and down or lightly tap the plate on the bench to resuspend the beads, then shake for another 5 minutes.

59. Place the **LNP** plate on the magnetic stand for 2 minutes or until the supernatant appears clear.
60. Using a multichannel pipette set to 30 µl, transfer the supernatant from the **LNP** plate to the **SGP** plate. Change tips between samples to avoid cross-contamination.
61. Seal the **SGP** plate with Microseal 'B' and then centrifuge at 1,000 xg for 1 minute.
62. If the **SGP** plate was stored frozen, thaw the **SGP** plate at room temperature.
63. Centrifuge the **SGP** plate at 1,000 xg for 1 minute at 20°C to collect condensation.
64. If the **SGP** plate was stored frozen, using a P200 multichannel pipette, mix each library to be sequenced by pipetting up and down 3–5 times. Change tips between samples.
65. Using a P20 multichannel pipette, transfer 5 µl of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip. Change tips after each column to avoid sample cross-contamination.
66. Label a fresh Eppendorf tube **PAL** (Pooled Amplicon Library).
67. Combine and transfer the contents of the PCR eight-tube strip into the **PAL** tube. Mix **PAL** well.
68. Label a fresh Eppendorf tube **DAL** (Diluted Amplicon Library)
69. Add 576 µl of Hybridization Buffer (HT1) to the DAL tube
70. Transfer 24 µl of **PAL** to the **DAL** tube containing HT1. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
71. Mix **DAL** by vortexing the tube at top speed.
72. Using a heat block, incubate the **DAL** tube at 96°C for 2 minutes.
73. After the incubation, invert **DAL** 1–2 times to mix and immediately place in the ice-water bath.
74. Keep **DAL** in the ice-water bath for 5 minutes.
75. Load **DAL** into a thawed MiSeq reagent cartridge into the **Load Samples** reservoir
76. Store the **PAL** tube and sealed **SGP** plate at -15° to -25°C for up to a week.
77. Sequence your library as indicated in the *MiSeq System User Guide*.